The Effects of Combination Antibiotic Therapy on Methicillin-Resistant *Staphylococcus aureus*

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Abstract

Antibiotics are becoming less effective as bacteria are acquiring resistance, so it is essential that new ones are discovered. An example of a multidrug-resistant bacterial strain is methicillin-resistant *Staphylococcus aureus* (MRSA), which has infected around 95,000 patients and caused 19,000 deaths per year in the United States alone. *Streptomyces* are soil bacteria that have been the source of many antibiotics and therapeutics. In order to potentially discover a novel antibiotic to treat resistant bacteria like MRSA, a *Streptomyces* was isolated from soil and determined to be *Streptomyces violaceorectus* based on its 16S rRNA gene sequence. *S. violaceorectus* produces an antibiotic that inhibits the growth of Gram-positive and Gram-negative bacteria. With the recent lack in antibiotic discovery, new treatment options are necessary to combat antibiotic resistance. Combination antibiotic therapy, the simultaneous use of multiple antibiotics, has become a successful clinical treatment, especially with the use of a beta-lactam antibiotic such as ampicillin.

This project helped determine the difference in inhibitory effect of the antibiotic produced by *S. violaceorectus* when used alone and in combination with ampicillin against MRSA. The antibiotic combination was more effective at inhibiting the growth of MRSA, and this difference was significant and replicable over 15 trials. Further analyses will increase our knowledge of the antibiotic produced by *S. violaceorectus* and its ability to inhibit resistant bacteria by itself or in combination with another antibiotic for future medical use.

Chapter 1: Literature Review

1.1: The History of Antibiotics

1.1.1: The Discovery of Penicillin

Sir Alexander Fleming was born in 1881 into a farming family in Scotland (1). During his childhood, Fleming's father died, which forced him and his siblings to determine their future career paths (1). He began work as an office clerk, but later switched to the field of medicine and joined the department of bacteriology at St. Mary's Hospital in London, England (1). Before leaving for a month-long vacation in the midst of his career, he set aside Petri dishes containing bacterial colonies of *Staphylococcus* (1). When he returned from his leave, Fleming noticed that one of his dishes was contaminated with a mold that had inhibited the growth of *Staphylococcus* (2) **(Figure 1-1)**. After taking samples of the mold, he conducted a series of experiments to determine the nature of its ability to inhibit bacteria. Fleming later identified the mold as *Penicillium* and named the inhibitory substance that it secreted penicillin (2). This accidental finding of his in 1928 eventually resulted in the discovery of the first true antibiotic, penicillin (3).

Bacterial infections that are easily treatable today were once a major cause of death. Before 1928, there were minimal treatment options for infected patients (1). This is a problem that Fleming wished to solve. Following the introduction of penicillin, he aimed to demonstrate if it could be used in human medicine. He began by performing experiments on animals, but at the time, most researchers were only using its

antimicrobial capabilities as a tool for isolating *Bacillus influenzae* (1). A few years later, Howard Florey and Ernst Chain were able to purify penicillin from cultures of Fleming's mold (3). This pure form of penicillin was manufactured and put to use during World War II as a treatment for soldiers with infectious diseases (1). Fleming, Florey, and Chain were later awarded with the Nobel Prize in Physiology or Medicine in 1945 for the significance of their discoveries in the field of microbiology (3).

Figure 1-1

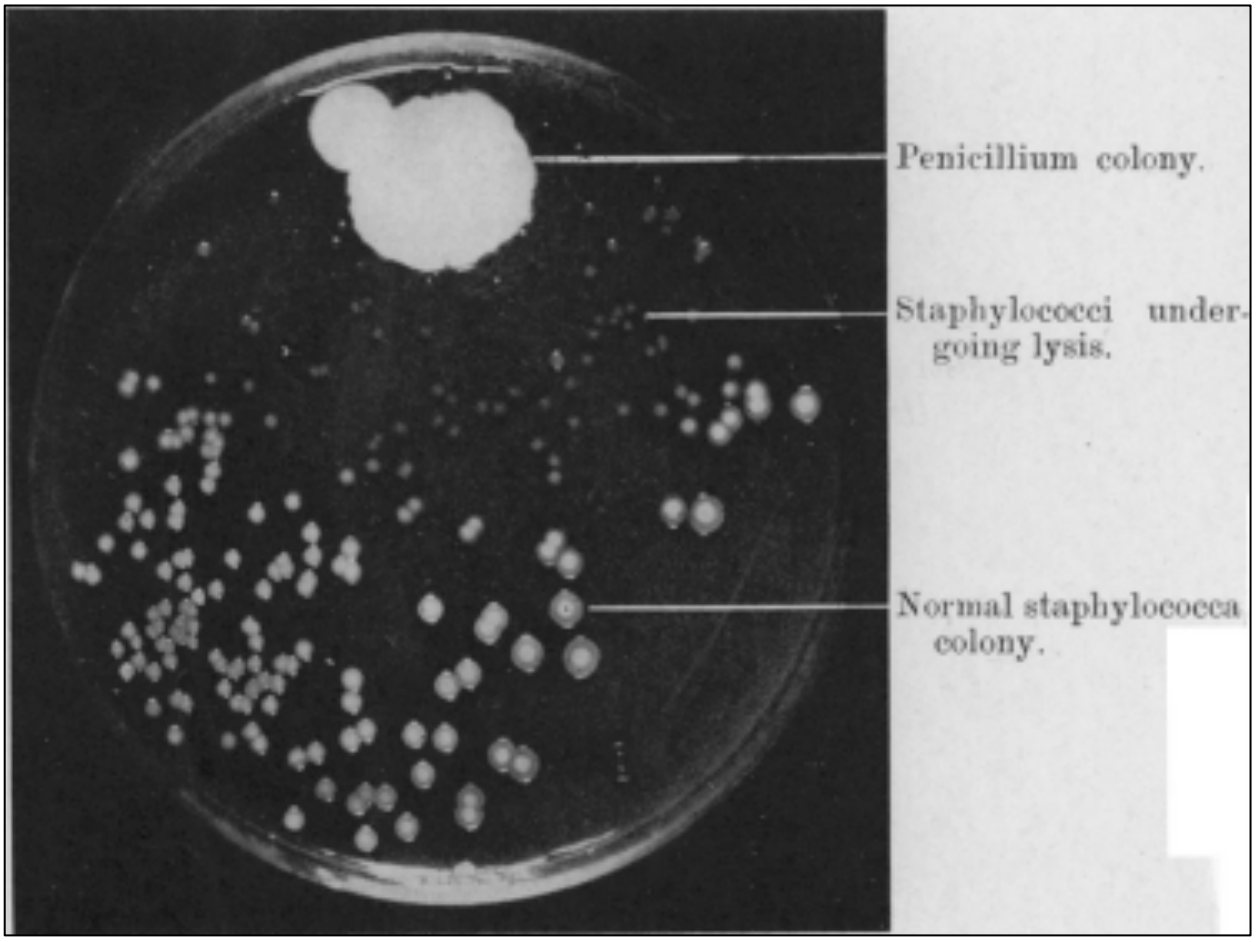

Figure 1-1. Inhibition of *Staphylococci* colonies due to *Penicillium*. A bacterial culture-plate displaying the dissolution of *Staphylococci* colonies in the presence of the fungi, *Penicillium.* Adapted from (2).

1.1.2: The Different Classes of Antibiotics

With the purification and identification of additional antibiotics, several systems were established in order to classify them. Most classification systems have organized antibiotics according to their molecular structures, mechanisms of action, and antimicrobial activity (4). There are multiple classes of antibiotics, and the following provides a brief description of the majority of them **(Figure 1-2):**

- Beta-lactams are antibiotics that contain a four-member ring moiety comprised of 3 carbons and 1 nitrogen (4) (a beta-lactam ring). The beta-lactam class is composed of four distinct antibiotic classes: Penicillins, Cephalosporins, Carbapenems, and Monobactams (5). These compounds interfere with peptidoglycan synthesis in the bacterial cell wall, which can lead to cell lysis/death (6). They do so by binding to penicillin-binding proteins (PBP), which are bacterial enzymes that assist in the formation of peptidoglycan (5). PBPs are primarily involved in the transglycosylation and cross-linking processes of peptidoglycan synthesis (7).
- Penicillins are beta-lactam antibiotics that contain a 6-animopenicillanic acid ring in their structure (4). Most of these compounds disrupt the synthesis of bacterial cell walls and have the suffix '–cillin' in their names (4). The Penicillin class includes antibiotics such as penicillin, amoxicillin, ampicillin, methicillin, and others (8).
- Cephalosporins are beta-lactam antibiotics that are similar to the penicillins in regards to their structure and mechanism of action. However, these compounds

contain a 7-animocephalosporanic acid ring in their structure (4). The Cephalosporin class includes antibiotics such as cephalexin, cefroxadine, cefadroxil, and others (9).

- Carbapenems are beta-lactam antibiotics that are frequently employed for patients infected with antibiotic-resistant bacteria (4). These compounds are able to resist beta-lactamases, enzymes that hydrolyze the beta-lactam ring of some antibiotics, due to the steric hindrance induced by the carbon atom at the C-1 position (10). They also inhibit the activity of beta-lactamases because of the *trans* configuration at the C-5 and C-6 position of their beta-lactam ring (10). The Carbapenems class includes antibiotics such as doripenem, imipenem, meropenem, and others (10).
- Monobactams are beta-lactam antibiotics with a structure that contains a beta-lactam ring that is not combined with another ring (4). The beta-lactam ring in these compounds stands alone. This structural variance allows for the attachment of different functional groups to the molecule and can inhibit the activity of beta-lactamases (11). The only available antibiotic in the Monobactam class is aztreonam, which was synthetically modified to enhance the activity of the beta-lactam ring when attempting to bind to its targets (11).
- Quinolones are antibiotics that inhibit bacterial DNA replication and transcription (4). These compounds generally have the suffix '–oxacin' in their names, and some modifications have been added to them to improve their antimicrobial potency (4). For example, a third ring added to their structure (4) or a halogen added to the 8-position of their fused rings enhances their activity against certain

anaerobic bacteria (12). The Quinolone class includes antibiotics such as norfloxacin, ofloxacin, cinoxacin, and others (12).

- Macrolides are antibiotics that contain a macrocyclic lactone ring in their structure that consists of 14-16 members (13). These compounds inhibit protein synthesis by binding to bacterial ribosomes and disrupting the formation of polypeptide chains (14). The Macrolide class includes antibiotics such as azithromycin, erythromycin, clarithromycin, and others (4).
- Tetracyclines are antibiotics that contain four hydrocarbon rings in their structure and have the suffix '–cycline' in their names (4). These compounds target the bacterial ribosome and disrupt protein synthesis (15). The Tetracycline class includes antibiotics such as tetracycline, oxytetracycine, chlortetracycline, and others (15).
- Sulphonamides are antibiotics that contain a sulfonamide group in their structure (16). At high concentrations, these compounds can kill bacteria (16). Otherwise, they prevent bacterial growth and reproduction. The Sulphonamide class includes antibiotics such as sulfacetamide, sulfadiazine, sulfamethoxazole, and others (17).
- Glycopeptides are antibiotics with a structure that contains a cyclic peptide core of seven amino acids that are bound to two amino sugars (18). The peptidic backbone of these compounds forms 5 hydrogen bonds when attaching to its target (19). The Glycopeptide class includes antibiotics such as vancomycin, teicoplanin, oritavancin, and others (18).

- Aminoglycosides are antibiotics with a structure that contains multiple glycosidic bonds that connect 3-amino sugars (20). These compounds disrupt bacterial protein synthesis by attaching to the A-site of ribosomal subunits and disturbing ribosomal translocation (21). The Aminoglycoside class includes antibiotics such as streptomycin, neomycin, gentamicin, tobramycin, and others (22).
- Oxazolidinones are synthetic antibiotics that contain 2-oxazolidone in their structure (23). These compounds interfere with protein synthesis by binding to the peptidyl transferase center of ribosomes (24). The Oxazolidinone class includes antibiotics such as linezolid, tedizolid, and others currently in clinical development (24).

Figure 1-2

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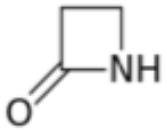

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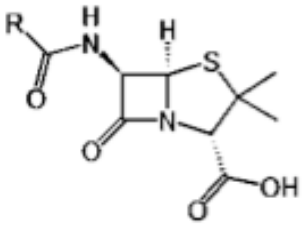

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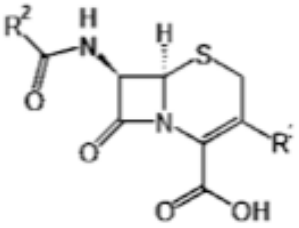

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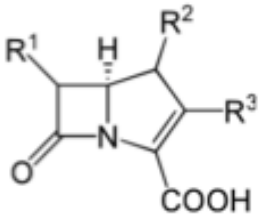

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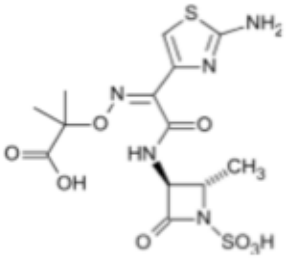

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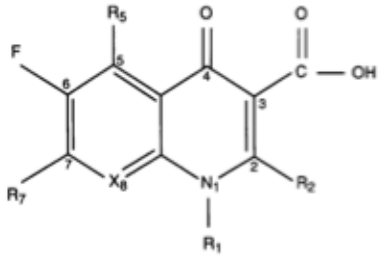

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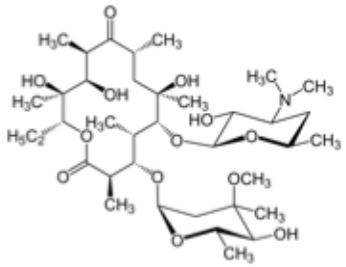

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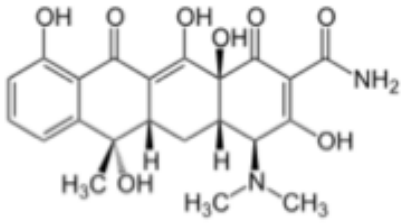

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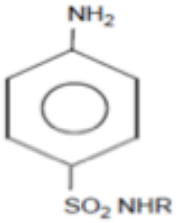

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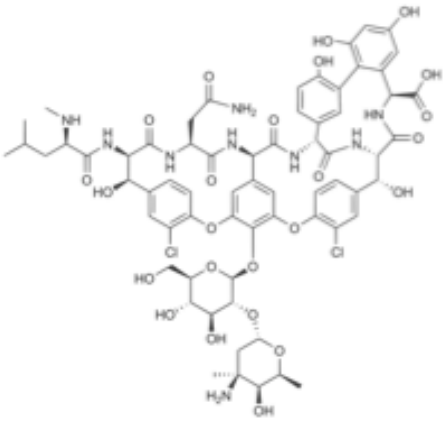

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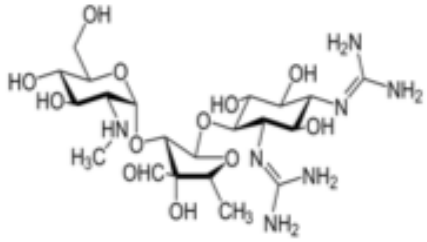

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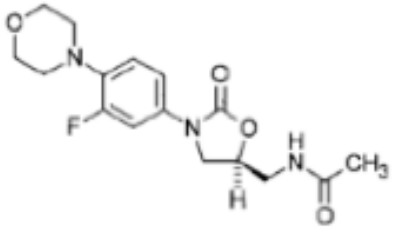


Figure 1-2. General structure of various antibiotic classes. A. Beta-lactam ring. **B.** Penicillins. **D.** Carbapenems. **C.** Cephalosporins. **E.** Monobactams. **F.** Quinolones. **G.** Macrolides. **H.** Tetracyclines. **I.** Sulphonamides. **J.** Glycopeptides. **K.** Aminoglycosides. **L.** Oxazolidinones. Structures adapted from (25, 26, 26, 10, 27, 28, 29, 30, 16, 18, 20, 23), respectively.

1.1.3: The Mechanisms of Action of Antibiotics

The different classes of antibiotics can affect bacteria through various mechanisms of action that occur inside a cell. These include the inhibition of bacterial protein synthesis, nucleic acid synthesis, and cell wall synthesis (4). Additionally, antibiotics can cause cell membrane breakdown in bacteria and can negatively affect bacterial metabolism by blocking fundamental metabolic pathways (4). These drugs generally target bacterial properties that are not present in or very different than eukaryotes, thus preventing harm to the host. Because of their mechanisms of action, the use of antibiotics commonly results in the termination of bacterial cell growth, and frequently cell death.

The bacterial ribosome, a molecular structure involved in protein synthesis, contains a 30S subunit and a 50S subunit that are comprised of rRNA and proteins (31). Together, these structures translate mRNA into amino acids through the initiation, elongation, and termination stages (31). The A-site on the 30S subunit is involved in codon-anticodon recognition, the peptidyl transferase center forms peptide bonds between adjacent amino acids that are attached to their corresponding tRNAs, and the exit tunnel on the 50S subunit allows the newly made peptide to leave the ribosome (31). Certain antibiotics, such as Aminoglycosides, bind to the A-site, prevent codon recognition, and interfere with translation (32). Oxazolidinones are compounds that bind to the peptidyl transferase center and inhibit the formation of peptide bonds (31). Macrolides are an example of an antibiotic that can disrupt protein synthesis by binding to and blocking the exit tunnel on the 50S ribosomal subunit to prevent protein growth

(31). These processes, carried out by several classes of antibiotics, lead to the termination of bacterial protein synthesis.

Nucleic acid synthesis is the process that produces new strands of bacterial DNA and RNA. DNA is synthesized through DNA replication, and RNA is synthesized through transcription. During both of these reactions, two key enzymes, topoisomerase II and topoisomerase IV, regulate DNA coiling (33). Their function is to overwind and underwind DNA strands to prevent them from breaking under the large amounts of tension created by helicases (33). Many antibiotics, such as Quinolones, bind to DNA-topoisomerase complexes and prevent the rejoining of the DNA or RNA strands (33). This results in the termination of nucleic acid synthesis. Other compounds can prevent the continuation of transcription by binding to and obstructing RNA polymerase, the enzyme that is involved in the formation of new RNA strands (32). Additionally, an antibiotic has been determined to inhibit the activity of DNA polymerase, the enzyme that is involved in the formation of new DNA strands (32). Regardless of their mechanism of action, the disruption of nucleic acid synthesis adversely affects bacterial cells.

The bacterial cell wall is an important structure that provides a cell with shape and resistance to various types of stress (5). It is comprised of a cross-linking network of peptidoglycan, a compound formed from the attachment of short peptides to N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) (5). The glycosidic and peptide bonds that form this large network of molecules are catalyzed with the assistance of penicillin-binding proteins (5). Most antibiotics that inhibit the synthesis of bacterial cell walls are Beta-lactams, and they do so by interacting with the active site of

penicillin-binding proteins (5). This prevents the formation of cross-links between peptidoglycan molecules, which eventually results in cell death. Other than Beta-lactams, the Glycopeptide antibiotic, vancomycin, has been determined to prevent the attachment of short peptides to NAM (34). Likewise, this mechanism of action can detrimentally affect bacterial cells.

The bacterial cell membrane acts like a barrier that selectively allows the passage of specific molecules in and out of a cell (35). It also has certain properties that make it an attractive target for many antibiotics. Cell membranes are primarily composed of phospholipids, but bacterial cell membranes have negatively charged lipids exposed on their surface (35). Cationic antibiotics can be used to bind to these anionic lipids to disrupt the clustering and curvature of the phospholipids (35). Compounds that affect these membrane properties can be toxic to bacteria. For example, Polymyxins are positively charged antibiotics that bind to lipopolysaccharide, a negatively charged component of the bacterial membrane (36). When this occurs, the outer layers of the cell destabilize, resulting in its death (36).

In addition to the mechanisms of action discussed, it has been determined that some antibiotics mimic specific molecules that are needed for key metabolic pathways (4). This would result in enzymes binding to these antibiotics instead of their proper substrates, causing various sorts of metabolic problems. For example, Sulphonamides mimic tetrahydrofolate, a compound required by bacterial cells for the synthesis of folic acid (4). Whether bacterial metabolism is negatively affected, nucleic acid or protein synthesis is inhibited, or the formation of the outer layers of cells is disrupted, antibiotics severely damage, and often kill, bacteria.

1.2: Antibiotic Resistance

1.2.1: The Rise of Antibiotic Resistance

The discovery of antibiotics has revolutionized the field of medicine and impacted healthcare across the globe. They have helped decrease the mortality rates of various regions, while ensuring the successful treatment of bacterial infections in patients (37). However, the efficacy of antibiotics has been shown to decrease over time due to the development of resistance in pathogenic bacteria, which occurs when bacteria acquire other genes or mutations that give them a competitive advantage in environments where there is selective pressure from antibiotic use (37). Multidrug-resistance occurs when bacteria acquire resistance to multiple classes of antibiotics, usually from the use of broad-spectrum antibiotics (38). Additionally, the multidrug-resistant capacity of numerous bacterial strains has spread through the processes of cross-infection and horizontal gene transfer (38). This has become a challenge for many countries and has resulted in higher morbidity rates worldwide (38). In order to combat the increasing rates of emergence of multidrug-resistant bacteria, new solutions are needed.

With the lack of clinical interventions available for patients infected with multidrug-resistant bacteria, the discovery of new antibiotics has appeared to be a plausible solution. However, it has been difficult to find compounds that can penetrate the bacterial cell membrane without being toxic to human cells (39). Another challenge has been generating antibiotics that are more successful than the current options used to treat infectious diseases (39). The production and development of candidate drugs is

also a long and costly process, so it may be at the best interest of the public health for business strategies to focus on minimizing the time and money needed for the implementation of clinical trials (39).

The “antibiotic resistance crisis” has become more problematic with the recent rise of multidrug-resistant bacteria and lack of antibiotic discovery. This has led to the introduction of multiple antibiotic development programs in the pharmaceutical industry (40). Given that it takes time for clinical trials to take place, some of the focus has shifted towards the prevention of the diffusion of multidrug-resistance pathogens and the improvement of the limited antimicrobial therapies that are currently being employed (37). Furthermore, new approaches to targeting bacterial infections can only be produced if the mechanisms of resistance and molecular pathways of antibiotic-resistant bacteria are fully understood (40).

1.2.2: Mechanisms of Resistance

When interacting with antibiotics, bacteria have been found to use various mechanisms of resistance to prevent any damage to their cell. These pathways are the result of intrinsic resistance or acquired resistance (41). Bacteria can be intrinsically resistant to different classes of antibiotics because of an inherent structural or functional property that obstructs the antibiotic’s mechanism of action (41). This is the reason why a specific antibiotic is not effective against all species of bacteria. Extrinsic resistance to antibiotics can develop in three ways: the prevention of access into the cell, the modification of antibiotic targets, and the inactivation of antibiotics (41). Acquired

resistance occurs as a result of mutations in bacterial genes or through the process of horizontal gene transfer (42).

In order to prevent an antibiotic from entering a bacterial cell, the bacterium can either reduce its membrane permeability or increase its efflux (41). The cell membrane is composed of multiple protein channels that permit the passage of different substances. These include porins and other non-selective channels, which allow for the passive diffusion of many particles, and selective channels, which allow for the diffusion of only specific particles (41). Bacteria can down-regulate the expression of non-selective protein channels and up-regulate the expression of more selective ones, thus restricting antibiotics from entering the cell (41). This can act as a cell's first line of defense against an antibiotic.

In addition to changing their cell membrane permeability, bacteria can increase the transport of antibiotics outside of the cell through the action of efflux pumps. Efflux pumps are active transporters on the cell membrane that move substances to the extracellular environment (42). Some of these molecular structures can transport a small range of substrates, while others, termed multidrug efflux pumps, can lead to the passage of a wide range of compounds out of the cell (43). Although all bacteria have genes that code for multidrug efflux pumps, they are only highly expressed in multidrug-resistant bacteria (41). Not only do they act on a variety of antibiotics because of their poly-substrate specificity, multidrug efflux pumps lower the intracellular concentration of antibiotics (43). Without antibiotics entering the cell, multidrug-resistant bacteria have a greater opportunity to acquire mutations that will confer additional resistance to other antibiotics.

Most antibiotics bind with high affinity to their intracellular targets (41). Bacteria have adapted to this by altering the target's molecular structure in such a way that still allows it to function normally (41). With a change in the structure of its target, an antibiotic cannot bind effectively. This usually occurs with the introduction of a mutation in the gene that codes for the target, which spreads throughout the bacterial population through recombination (41). Bacteria have also developed protective mechanisms to prevent efficient antibiotic binding. For example, Aminoglycoside antibiotics are inhibited from binding to their target, the ribosome, if it is methylated (41). Additionally, some intracellular compounds have been discovered to promote the release of an antibiotic from an antibiotic-target complex (41). Even though the antibiotic might have entered the cell in either case, it will not be able to exert its effects because it cannot properly bind to its target.

It has also been determined that bacteria can block the action of antibiotics by degrading or modifying them (42). There are thousands of bacterial enzymes that can destroy foreign compounds through the process of hydrolysis. For example, the use of penicillin led to the discovery a penicillinase, a beta-lactamase (42). Beta-lactamases are hydrolytic enzymes expressed in bacteria that degrade beta-lactam antibiotics (10). Likewise, there are compounds known as extended-spectrum beta-lactamases, which can act on all classes of beta-lactam antibiotics (42). Additionally, bacterial enzymes can add chemical groups, such as phosphate, acyl, and nucleotidyl groups, to an antimicrobial substance (41). This ensures that the modified antibiotic will be sterically hindered from binding to its target.

Whether bacteria acquire resistance or are intrinsically resistant to antibiotics, there is a diverse array of mechanisms that can confer antibiotic resistance. It is possible that more are discovered as new approaches are utilized to treat infectious diseases in patients.

1.2.3: How Antibiotic Resistance Spreads

When a bacterium acquires a mutation in a gene that confers antibiotic resistance, it will have a better chance of survival than susceptible bacteria in an environment with antibiotics (42). In a population of bacteria, this antibiotic-resistant mutant will predominate and reproduce while antibiotic-susceptible bacteria die.

Bacteria can also obtain genetic material from methods other than reproduction, such as through horizontal gene transfer (44). This includes the processes of transformation, transduction, and conjugation (42). Transformation occurs when bacteria directly uptake foreign DNA or a replicable plasmid from their surrounding environment and integrate it into their genome (45). Transduction occurs when bacteriophages infect a bacterial cell and introduce foreign DNA into its genome (41). Conjugation occurs when a cell-to-cell junction is formed between two bacterial cells, and one of them transfers some of its DNA to the other (42). Most often, mobile genetic elements, such as transposons and plasmids, are replicated and then transported into the conjugated cell (42). The genetic material that is passed between bacteria as a result of horizontal gene transfer can contain genes that confer antibiotic resistance. When this is the case, as it is often, antibiotic resistance spreads.

1.2.4: Methicillin-Resistant *Staphylococcus aureus*

Staphylococcus aureus is a facultative aerobic strain of Gram-positive bacteria that has infected humans and animals for centuries (7). As opportunistic pathogens, they most commonly infect soft tissue, the skin, and the respiratory system (7). *S. aureus* also naturally colonizes and lives in various parts of the human body (7). When penicillin was first introduced in the 1940s, it became the first antimicrobial substance that was effective against *S. aureus* (1). Only one year later, penicillin-resistant *S. aureus* evolved and was found to contain a plasmid with the beta-lactamase gene, *blaZ*, which conferred antibiotic resistance (7). In 1959, methicillin, a derivative of penicillin, was used to successfully treat patients infected with penicillin-resistant *S. aureus* (7). Similarly, methicillin-resistant *S. aureus* (MRSA) evolved two years later (7). Since then, MRSA has continued to be a significant problem to the public health of regions across the globe, severely impacting their morbidity and mortality rates (46). New treatment options for patients infected with these difficult-to-treat multidrug-resistant bacteria must be found.

The cell wall of MRSA has a thick layer of peptidoglycan, which is cross-linked in a reaction catalyzed by various penicillin-binding proteins (PBPs) (7). Most PBPs have two domains, one involved in transglycosylation and one involved in the cross-linking process (7). Methicillin and other beta-lactam antibiotics bind to and inhibit the cross-linking domain of PBPs, thus disrupting the formation of peptidoglycan in *S. aureus* and killing the cell (5). However, MRSA has evolved the use of a different PBP, PBP2a, which has a reduced affinity for beta-lactam antibiotics (46). The *mecA* gene encodes

PBP2a, and therefore, confers resistance to beta-lactam antibiotics, including methicillin (7). The mobile chromosomal cassettes in MRSA known as Staphylococcal chromosomal cassettes *mecA* (SCC*mecA*) contain the *mecA* gene (46). It has been hypothesized that the *mecA* gene was acquired through horizontal gene transfer from a different species of *Staphylococcus* (46).

Strains of MRSA have been differentiated into two broad groups: hospital-acquired MRSA (HA-MRSA) and community-acquired MRSA (CA-MRSA) (47). These pathogenic forms are genetically and epidemiologically distinct (47). Initially, MRSA infections would emerge as HA-MRSA in major hospitals and healthcare systems, where they are now considered endemic (47). The pathogen would spread through contact between patients, physicians, and other individuals working those environments. But in the 1990s, CA-MRSA clones that differed from HA-MRSA were found in healthy people in the community who had no prior healthcare exposure (46). Although both forms of MRSA are resistant to beta-lactam antibiotics, they are treated differently. Variations in treatment also depend on the location, severity, and type of infection (47). For example, CA-MRSA strains are most commonly treated with vancomycin, tetracycline, and clindamycin (7). HA-MRSA strains are also susceptible to vancomycin, but can effectively be treated with daptomycin and linezolid too (7). However, the difference between HA-MRSA and CA-MRSA is being blurred, as they are both rapidly acquiring resistance to the same antibiotics (47).

The incidence of MRSA infections has increased worldwide, and its range of distribution is estimated to be between 23% and 73% (7). In the United States, the prevalence of MRSA has also been increasing, from 2% in 1974 to 64% in 2004 (7). It

has been determined that MRSA infects about 95,000 patients and causes 19,000 deaths per year in the U.S. alone (46). There are many factors that can assist in the transmission of this pathogen, such as a compromised immune system (47). Likewise, crowded environments, skin-to-skin contact, lack of cleanliness, and contamination can also influence MRSA's highly virulent path (47). The spread of MRSA is a public hazard that needs to end, especially since treatment options for infected patients are diminishing as these bacteria are rapidly evolving resistance to additional antibiotics.

1.3: *Streptomyces*

1.3.1: Selman Waksman

Selman Abraham Waksman was born in Novaya Priluka, Ukraine towards the end of the 19th century (48). As a child, he became very interested in the fertile soil that surrounded his hometown. He later migrated to the United States and attended Rutgers College, where he studied soil bacteria (48). Specifically, Waksman focused on actinomycetes, which are Gram-positive, facultative anaerobic bacteria (48). After noticing how actinomycetes multiply and kill common bacteria also living in the soil, he began to consider the possibility of antibiotic production (49).

Waksman and his research team were persistent in their studies of actinomycetes, resulting in the discovery of four new antibiotics: actinomycin, fumigacin, streptothricin, and clavacin (49). Unfortunately, tests indicated that the antibiotics displayed toxicity in animals (49). Waksman continued to search for more antibiotic-producing soil bacteria, and in 1943, he isolated a species of bacteria that resembled *Actinomyces griseus* (49). However, unlike previous *A. griseus* isolates, this new isolate produced an antibiotic (49). Waksman termed the new antibiotic, streptomycin, and the new species of bacteria, *Streptomyces griseus* (49). This resulted in the creation of a new genus of actinomycete bacteria, *Streptomyces.*

Streptomycin was found to be the first antibiotic that effectively cured tuberculosis, significantly impacting the field of medicine (49). Waksman also successfully received a patent for the antibiotic and helped develop a fermentation

method for its mass production (48). He became known as the “Father of Antibiotics” and was awarded with the Nobel Prize in Physiology or Medicine in 1952 (48). Notably, Waksman revolutionized the field of microbiology with his discovery of the *Streptomyces* genus.

1.3.2: *Streptomyces*: A Possible Solution to Antibiotic Resistance

Streptomyces are Gram-positive, filamentous bacteria that generally inhabit soil and are involved in the decomposition of organic matter (50). As phylogenetically part of the Actinobacteria phylum, their DNA is GC-rich (50). To survive stressors or extreme environments, some species of *Streptomyces* can produce exospores. In favorable conditions, spores germinate to form hyphae, a structure commonly found in fungi but rare among most Gram-positive bacteria (50). Together, hyphae filaments arrange into interlinking networks known as mycelium. *Streptomyces* are also known to produce secondary metabolites such as antifungals and antibiotics, which has made them an important focus of study in the treatment of bacterial infections (50). The most studied species of *Streptomyces* are *Streptomyces griseus,* for the production of streptomycin, and *Streptomyces coelicolor,* for genetic analyses (50).

Streptomyces are abundant and adaptable bacteria with high rates of secondary metabolite production. Antibiotic production is species specific throughout the entire genus, and antibiotic secretion enables *Streptomyces* to compete with other microorganisms living in the surrounding soil (50). Antibiotics are also beneficial to plants by protecting them from harmful bacteria, creating a symbiotic relationship

between certain species of *Streptomyces* and plants living in the same environment (50). Furthermore, the genetic sequencing of these bacteria can be used to indicate the genetic basis and purpose of their antibiotic production.

Streptomyces have produced 80% of the antibiotics that are currently identified, many of which are utilized in clinical practice (50). These antibiotics, such as streptomycin, tetracycline, vancomycin, and daptomycin, can be and have been used to combat multidrug-resistant bacteria (50). However, with the recent lack in antibiotic discovery, infectious diseases continue to be one of the leading causes of death globally (50). As bacterial infections remain the cause of millions of deaths, the search for new antibiotics is essential. Nonetheless, *Streptomyces* might be the source of a solution to this ongoing crisis.

1.4: Combination Antibiotic Therapy

1.4.1: The Positive/Negative Effects of Combination Antibiotic Therapy

With the recent rise of multidrug-resistant bacterial infections, the necessity for new treatment options for patients is vital. One such possibility that has been employed is combination antibiotic therapy, a treatment that involves prescribing multiple antibiotics to be taken simultaneously (51). Combination antibiotic therapy is a highly selective process, but it frequently consists of the use of a beta-lactam antibiotic with an aminoglycoside antibiotic (51). Whether this creates more or less success for infected patients has become a subject of debate, particularly because combination antibiotic therapy has been determined to only work in certain cases. However, the advantages and disadvantages of combination antibiotic therapy can assist in deciding the best situations for its utilization.

One of the main reasons for the usage of combination antibiotic therapy is to create synergistic drug interactions (51). Synergistic combinations of antibiotics can kill multidrug-resistant infections more effectively than antibiotics that are taken individually (51). For example, it was determined that the combination of *Stephania suberosa* Forman extract and ampicillin displays synergy at inhibiting the growth of ampicillin-resistant *Staphylococcus aureus* (52). It was also determined that the combination of a bioactive fraction from *Duabanga grandiflora* and ampicillin displays synergy at inhibiting the growth of MRSA (53). Additionally, the combination of tigecycline and carbapenem has been used to effectively treat patients with carbapenemase-containing

Klebsiella pneumonia infections (51), and the combination of ceftazidime and tobramycin has been proven to be an effective treatment for cystic fibrosis patients infected with *Pseudomonas aeruginosa* (54). Antibiotic combinations broaden the antimicrobial spectrum, ensuring that at least one antibiotic will be successful at treating the infected patient. This can work especially well on polymicrobial infections, diseases that contain multiple bacterial pathogens (51). Likewise, combination antibiotic therapy can help prevent the emergence of bacterial resistance by forcing bacteria to acquire mutations that confer resistance to more than just one antibiotic in order to survive (51). This does not occur as often as singular drug resistance, which is why combination antibiotic therapy may be advantageous.

However, combination antibiotic therapy is a costly treatment that may put patients at risk for adverse side effects. Unlike synergistic drug interactions, certain combinations might display antagonism, where one antibiotic can either nullify or weaken the effects of the other on bacteria (54). When this occurs, the antibiotic combination will be less effective at treating patients than if each antibiotic was used individually. For example, it was determined that the combination of ceftazidime and ciprofloxacin was less effective at inhibiting the growth of *Pseudomonas aeruginosa* than when each was used alone (55). The potency of antagonism depends on the situation, but the worst-case scenario would be the development of a superinfection (54). Combinations of antibiotics frequently cannot kill every bacterial cell, because certain cells will acquire mutations that enable them to survive in an antimicrobial environment. Concurrently, other nearby bacteria might also develop resistance and could potentially add to the previous infection. This creates what is known as a

superinfection, where different pathogens that are resistant to the antibiotics infect the same patient (54). In order to prevent these unfavorable consequences of combination antibiotic therapy, physicians should ensure that its benefits outweigh the costs on a circumstantial basis.

1.4.2: Drug Interactions

There has recently been a lag in the discovery of antibiotics and great difficulty in selecting effective drug combinations. As the treatment options for patients with bacterial infections shifts more towards combination antibiotic therapy, so does the study of drug interactions. It is important to consider how different antibiotics will react with one another to find the most successful antibiotic combinations. Antibiotics can interact in an additive, synergistic, antagonistic, or suppressive manner (56). Additive drug combinations are just as effective at inhibiting bacterial growth as the sum of each antibiotic when used alone (56). In comparison to additive combinations, synergistic combinations are more effective and antagonistic combinations are less effective (56). Suppression is simply an extreme version of antagonism where an antibiotic combination is less effective against bacteria than the use of just one of the antibiotics (56).

Current research has focused on discovering synergistic antibiotic combinations, and approaches such as the chequerboard technique (57) and INDIGO (*INferring Drug Interactions using chemo-Genomics and Orthology*) (58) have assisted in doing so. Chequerboards and time-kill curves are used to set fractional inhibitory concentration

indices for determining synergy or antagonism amongst antibiotic combinations (57). This method can be helpful by comparing new data with already published results. INDIGO also predicts effective drug interactions, but consists of an algorithm that uses chemogenomics to systematically screen for antibiotic combinations (58). When experimentally tested, INDIGO was able to identify synergistic and antagonistic drug combinations that are effective against *Escherichia coli* and *Staphylococcus aureus* (58). The data has suggested that antagonistic combinations are more prevalent than synergistic combinations (58). Furthermore, drug interactions vary in their effectiveness against different bacteria.

1.4.3: Antibiotic Stewardship

Antibiotic stewardship refers to the proposed interventions that are aimed at improving the appropriate use of antibiotics and selecting for the ideal drug dosage, duration, and administration (59). The Infectious Diseases Society of America, the Pediatric Infectious Diseases Society, and the Society for Healthcare Epidemiology of America have defined this as the pathway to superior patient care and reduced risk of adverse side effects from antibiotic utilization (59). Many hospitals have incorporated an antibiotic stewardship program as a guideline for physicians who are prescribing antibiotics to their patients (40). For example, it is recommended that alternative dosing strategies are used over broad-spectrum beta-lactam antibiotics, physicians increase the appropriate use of and transition to oral antibiotics, antibiotic therapies are reduced to the shortest duration of time, and antibiotic cycling is not performed (59). Antibiotic

stewardship programs should be implemented in all accredited healthcare facilities and need to adapt to the local environments of their patient base. With more people receiving an evidence-based education on antibiotic usage, healthcare systems can find cost-effective strategies to prevent the emergence of antibiotic resistance in infected patients.

Chapter 2: Research Manuscript

2.1: Introduction

Since the discovery of penicillin by Alexander Fleming in 1928 (1), antibiotics have become an important solution to various medical complications. For example, bacterial infections that previously resulted in millions of deaths can now be cured with a simple, prescribed medication. However, as antibiotic use increased, the emergence of antibiotic-resistant bacteria also increased (38). Resistance is acquired with the accumulation of genes that confer resistance through specific agents, multidrug efflux pumps, and other factors (60). This rise in antibiotic-resistant bacterial infections has decreased the efficacy of antibiotics currently used to treat infected patients, which has proven to be detrimental to the overall health of society (61). An example of a multidrug-resistant bacterial strain is methicillin-resistant *Staphylococcus aureus* (MRSA), which are Gram-positive bacteria that have infected approximately 95,000 patients and caused 19,000 deaths per year in the United States alone (46). The incidence of MRSA infections has increased worldwide, and its range of distribution is estimated to be between 23% and 73%, thus creating a hazard to the public health (7).

As treatment options are diminishing, it is essential that new antibiotics are discovered. Unfortunately, the search for new antibiotics has been impeded because of the high costs associated with testing the success of new drugs (61). A genus of Gram-positive bacteria, *Streptomyces*, could end this antibiotic resistance crisis. *Streptomyces* were discovered by Selman Waksman in 1943 and are primarily found in soil (49). They

produce diverse secondary metabolites that have historically been a source of antibiotics and antifungals (50). Additionally, *Streptomyces* have produced 80% of the antibiotics currently used in clinical practice (50). *Streptomyces violaceorectus* is a Gram-positive bacterium isolated from the University of Texas at Austin that is able to produce an antimicrobial substance that is effective against Gram-positive bacteria, Gram-negative bacteria, and fungi (62). The strength of its effect on each varies, with it being the most effective against Gram-positive bacteria (62). The antibiotic produced by *S. violaceorectus* resembles endomycin, an antibiotic used in medical treatment, so it could have future applications in human medicine (62).

Combination antibiotic therapy is a common practice in which multiple antibiotics are prescribed together for simultaneous use to treat a bacterial infection (63). This is significant because the combined effect of the antibiotics can overcome the resistance of bacteria to a single antibiotic (63). Antibiotics in combination can target different essential processes of a bacterium, such as preventing protein synthesis or destroying cell walls, which can eventually lead to cell death (4). Combination antibiotic therapy with a beta-lactam antibiotic, such as ampicillin, is commonly used as a clinical treatment for patients dealing with resistant bacterial infections (64).

Little is known about the antibiotic produced by *S. violaceorectus*, and it is important to understand how it interacts when combined with ampicillin against antibiotic-resistant bacteria. The experiments described in this manuscript tested the effectiveness of combination antibiotic therapy using the antibiotic produced by *S. violaceorectus* and ampicillin against MRSA. Specifically, I measured the difference in inhibitory effect of the antibiotic produced by *S. violaceorectus* when used alone and in

combination with ampicillin on MRSA. I also measured the difference in inhibitory effect of the antibiotic produced by *S. violaceorectus* when used alone and in combination with ampicillin on methicillin-sensitive *Staphylococcus aureus* (MSSA) to control for the difference between MRSA and MSSA. Results indicated that the antibiotic combination was significantly more effective at inhibiting the growth of both MRSA and MSSA.

2.2: Methods

2.2.1: Strains, Media, and Growth Conditions

Tryptic Soy Broth Yeast Extract (TSB-YE) agar (30 g tryptic soy broth, 5 g yeast extract, 20 g agar, and 1 L nanopure water) was used for the growth of *S. violaceorectus.* TSB-YE agar was also used for the growth of MRSA and MSSA during all disc assay experiments. MRSA LAC-CI3 and MSSA HG003-CI48 strains were obtained from Dr. Marvin Whiteley. All agar plates were grown at room temperature for at least four days. *S. violaceorectus* colonies were grown in 25 mL of TSB-YE media for four days in a 30° C shaking incubator to be used for ethyl acetate extraction.

2.2.2: Bacterial Strain Isolation and Identification

Soil samples were collected at the University of Texas at Austin and plated on Actinomycete Isolation agar (20 mL 50% glycerol, 2.5 g L-arginine, 1 g NaCl, 0.1 g $CaCO_3$, 10 mg $FeSO_4$ - $7H_2O$, 10 mg $MgSO_4$ – $7H_2O$, 20 g agar, 200 mg cyclohexamide in EtOH, and 1 L nanopure water). Bacteria were isolated from Actinomycete Isolation agar and plated on TSB-YE agar for growth. The 16S rRNA gene of strains was amplified through polymerase chain reaction using universal 16S primers (forward – AGAGTTTGATCCTGGCTCAG and reverse – ACTACCAGGGTATTAATCC) and the Thermo Taq polymerase system. PCR products were sequenced at the University of

Texas Institute for Cell and Molecular Biology DNA core facility. Strains were identified through Basic Local Alignment Search Tool (BLAST) analysis.

2.2.3: Ethyl acetate Extractions

20 mL of *S. violaceorectus* cultures were mixed with an equal volume of ethyl acetate, vortexed for 60 seconds, and allowed to phase separate. Once separated, the organic layer was removed and this process was repeated. The extracts were evaporated to dryness under a constant stream of nitrogen gas. Extracts were then re-suspended in 200 µL of methanol. All extracts were combined into a single glass vial to create uniformity for disc assays.

2.2.4: Disc Assays

A lawn of MRSA (or MSSA) was spread on a TSB-YE agar plate. A paper disc was saturated in the extract of *S. violaceorectus* and dried for 60 seconds. Once dry, it was placed on the bacterial lawn. This procedure was repeated using methanol (instead of the extract of *S. violaceorectus*) to act as a control. To display combination antibiotic therapy, the same procedure was repeated using the extract of *S. violaceorectus*, except 2 µL of 100 µg/mL ampicillin (dissolved in water) were added to the disc after it was placed on the lawn. To control for ampicillin, a disc was placed on the lawn and 2 µL of 100 µg/mL ampicillin were added to it. The diameters of the zones of inhibition on disc assays were measured after one week of growth to test the inhibitory effect of the

antibiotic produced by *S. violaceorectus* under different conditions (larger zones represented greater inhibition). They were used to measure the difference in inhibitory effect of the antibiotic produced by *S. violaceorectus* when used alone and in combination with ampicillin on MRSA. They were also used to measure the difference in inhibitory effect of the antibiotic produced by *S. violaceorectus* when used alone and in combination with ampicillin on MSSA to control for the difference between MRSA and MSSA. Disc assays were replicated for 15 trials, and the results were analyzed with a two-sample t-test.

2.3: Results & Discussion

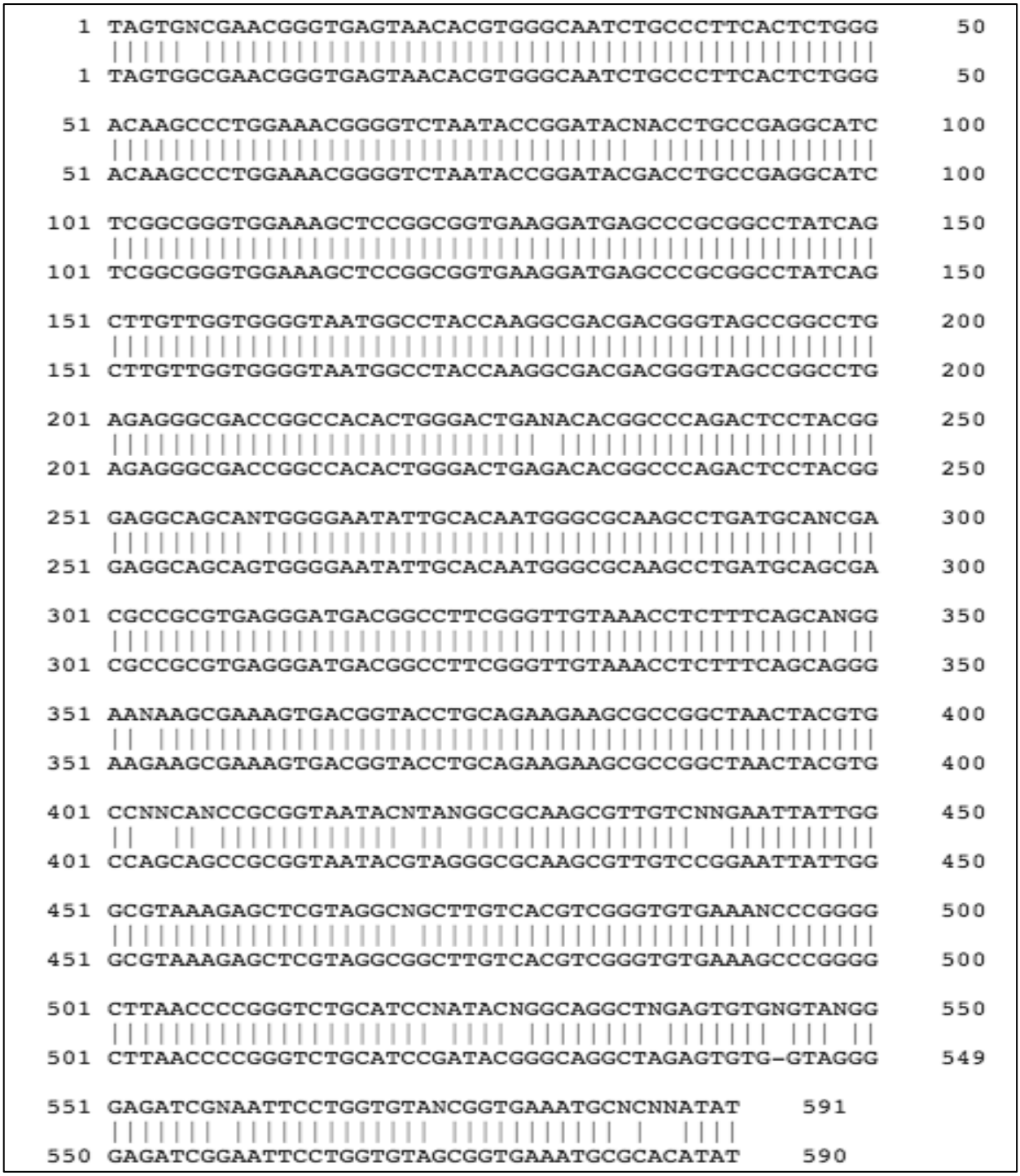
2.3.1: Isolation of Bacteria

In order to potentially discover new antibiotic-producing bacteria, a strain of *Streptomyces* was isolated from soil at the University of Texas at Austin and its 16S rRNA gene was amplified and sequenced. BLAST analysis determined its closest relative to be *Streptomyces violaceorectus* **(Figure 2-1A)**. *S. violaceorectus* produces an antimicrobial substance that inhibits the growth of Gram-positive bacteria, Gram-negative bacteria, and fungi (62). *Streptomyces* have produced many of the antibiotics used in clinical practice (50), so there is a possibility that this unknown compound could have future applications in human medicine.

There remains a necessity for the discovery of new antibiotics and new treatment options for patients coping with antibiotic-resistant infections because of the ongoing antibiotic resistance crisis. With minimal research on *S. violaceorectus*, any information regarding the antibiotic that it produces could be pertinent to the medical field.

Figure 2-1

A.


1 TAGTGNCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTTCACTCTGGG 50
1 TAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTTCACTCTGGG 50
51 ACAAGCCCTGGAAACGGGGTCTAATACCGGATACNACCTGCCGAGGCATC 100
51 ACAAGCCCTGGAAACGGGGTCTAATACCGGATACGACCTGCCGAGGCATC 100
101 TCGGCGGGTGGAAAGCTCCGGCGGTGAAGGATGAGCCCGCGGCCTATCAG 150
101 TCGGCGGGTGGAAAGCTCCGGCGGTGAAGGATGAGCCCGCGGCCTATCAG 150
151 CTTGTTGGTGGGGTAATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTG 200
151 CTTGTTGGTGGGGTAATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTG 200
201 AGAGGGCGACCGGCCACACTGGGACTGANACACGGCCCAGACTCCTACGG 250
201 AGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGG 250
251 GAGGCAGCANTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCANCGA 300
251 GAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGA 300
301 CGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCANGG 350
301 CGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGG 350
351 AANAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTG 400
351 AAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTG 400
401 CCNNCANCCGCGGTAATACNTANGGCGCAAGCGTTGTCNNGAATTATTGG 450
401 CCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGG 450
451 GCGTAAAGAGCTCGTAGGCNGCTTGTCACGTCGGGTGTGAAANCCCGGGG 500
451 GCGTAAAGAGCTCGTAGGCGGCTTGTCACGTCGGGTGTGAAAGCCCGGGG 500
501 CTTAACCCCGGGTCTGCATCCNATACNGGCAGGCTNGAGTGTGNGTANGG 550
501 CTTAACCCCGGGTCTGCATCCGATACGGGCAGGCTAGAGTGTG-GTAGGG 549
551 GAGATCGNAATTCCTGGTGTANCGGTGAAATGCNCNNATAT 591
550 GAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCACATAT 590

B.

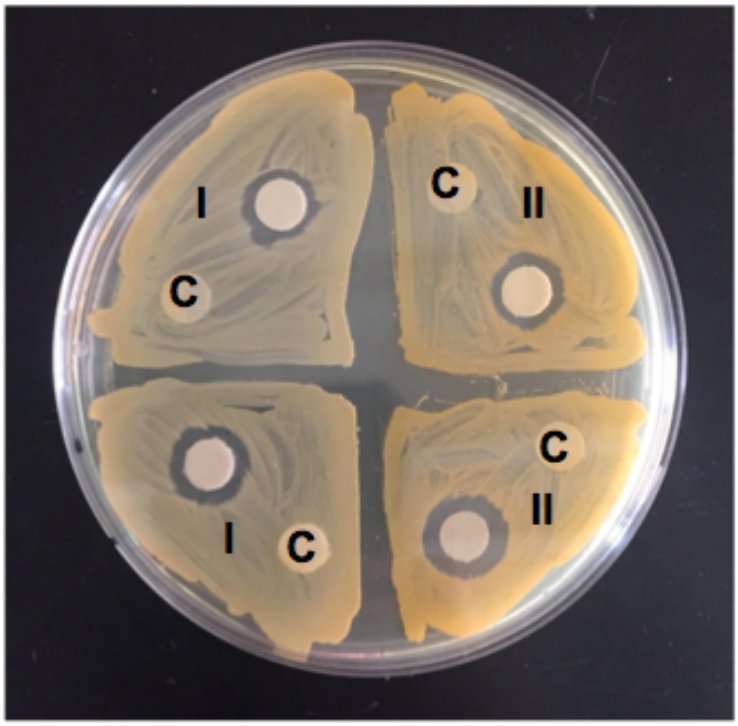


Figure 2-1. Characterization of an antibiotic-producing Streptomycete. An antibiotic-producing Streptomycete was isolated from soil on the University of Texas at Austin campus. Its 16S rRNA gene was sequenced and BLAST analysis suggested *S. violaceorectus* to be the most closely related strain. **A.** Pairwise sequence alignment of isolate (top sequence) and *S. violaceorectus* (bottom sequence). **B.** The ability of an extract of *S. violaceorectus* to inhibit the growth of MRSA and MSSA was demonstrated by zones of inhibition. The symbol "**I**" represents a bacterial lawn of MRSA, and the symbol "**II**" represents a bacterial lawn of MSSA. The symbol "**C**" represents the methanol control.

2.3.2: Organic Extractions

In order to isolate the antibiotic produced by *S. violaceorectus*, organic extractions of *S. violaceorectus* cultures were performed. During the growth of bacterial cultures, many cells secrete compounds such as antimicrobials and antifungals. Organic extractions are necessary to remove impurities and contaminants. Multiple extracts of *S. violaceorectus* were prepared and combined together. This created uniformity and controlled for the variance between different extractions. *S. violaceorectus* extracts were used to test their inhibitory effect on MSSA and MRSA through combination antibiotic therapy with ampicillin.

2.3.3: Inhibition of MSSA and MRSA

In order to determine if *S. violaceorectus* extracts have antimicrobial properties, a disc assay was performed to demonstrate that the extract from *S. violaceorectus* cultures inhibits the growth of both MSSA and MRSA **(Figure 2-1B)**. Although MRSA exhibits resistance to multiple antibiotics, this result confirms the effectiveness of *S. violaceorectus* extracts at inhibiting its growth. With MRSA infecting hundreds of thousands of individuals worldwide (46), new methods need to be developed to end its nature as a hazard to the public health. The success of the antibiotic produced by *S. violaceorectus* against MRSA makes it a promising candidate for the pharmaceutical industry as a potential clinical drug.

A separate disc assay was performed to represent the inhibitory effects of combination antibiotic therapy on MRSA and MSSA **(Figure 2-2)**. It demonstrates that the antibiotic produced by *S. violaceorectus* also inhibits the growth of both MSSA and MRSA when combined with ampicillin. This finding is critical because new treatment options are needed for patients who have multidrug-resistant bacterial infections. One such treatment is combination antibiotic therapy. The results from this disc assay prove that the antibiotic produced by *S. violaceorectus* is a promising candidate for this type of treatment. Additionally, combination antibiotic therapy with *S. violaceorectus* extracts might display superiority to currently used antibiotic combinations, which would be beneficial to patients dealing with difficult-to-kill bacterial infections.

Figure 2-2

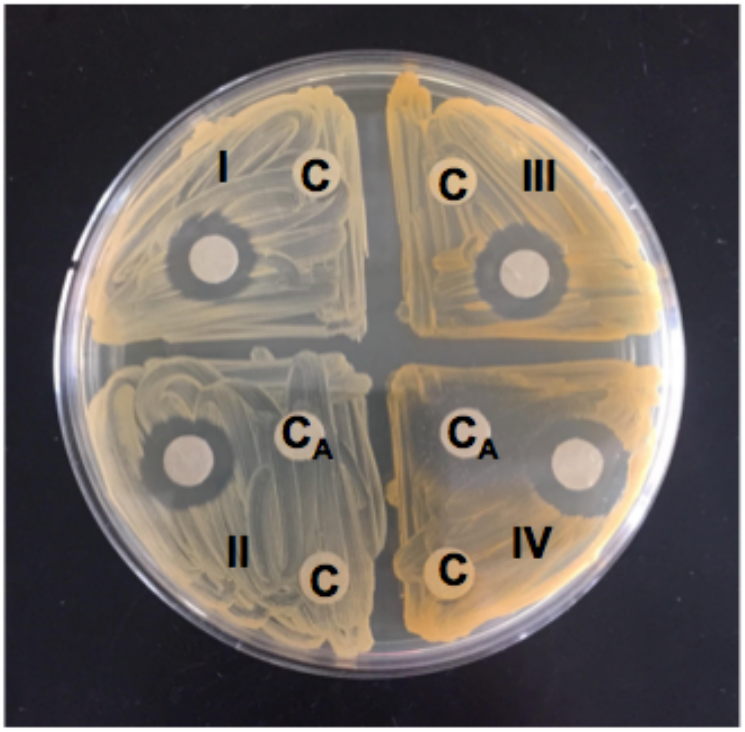


Figure 2-2. Representative disc assay. Disc assay to represent all factors of the experiment. The symbol "**I**" represents a bacterial lawn of MRSA, and demonstrated is the ability of an extract of *S. violaceorectus* to inhibit the growth of MRSA. The symbol "**II**" represents a bacterial lawn of MRSA, and demonstrated is the ability of the combination of an extract of *S. violaceorectus* and ampicillin to inhibit the growth of MRSA. The symbol "**III**" represents a bacterial lawn of MSSA, and demonstrated is the ability of an extract of *S. violaceorectus* to inhibit the growth of MSSA. The symbol "**IV**" represents a bacterial lawn of MSSA, and demonstrated is the ability of the combination of an extract of *S. violaceorectus* and ampicillin to inhibit the growth of MSSA. The symbol "**C**" represents the methanol control, and the symbol "$\mathbf{C_A}$" represents the ampicillin control. Note that the ampicillin control inhibits the growth of MSSA and not MRSA, indicating that MRSA is resistant to ampicillin.

2.3.4: Inhibitory effect of the antibiotic produced by *S. violaceorectus* when used alone vs. in combination with ampicillin on MRSA

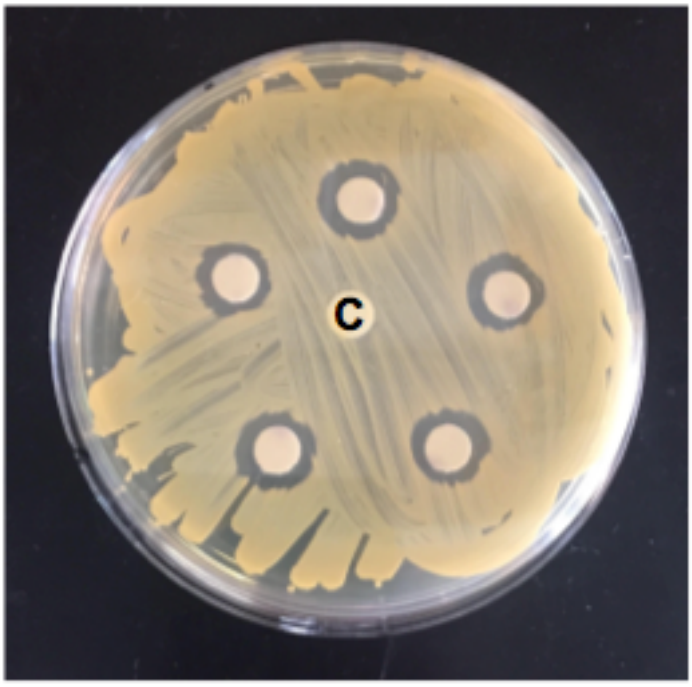
In order to determine if the diameters of the zones of inhibition on disc assays were larger when the antibiotic produced by *S. violaceorectus* was used in combination with ampicillin than when used alone on MRSA, a two-sample t-test using the diameters from 15 disc assays was performed **(Figure 2-3)**. This result was significant with a p-value less than 0.00001 and demonstrates that the antibiotic combination had a greater inhibitory effect on MRSA. Similar results were obtained when the antibiotic produced by *S. violaceorectus* was used in combination with ampicillin against MSSA **(Figure 2-4)**. The addition of ampicillin to the antibiotic produced by *S. violaceorectus* increased the diameters of zones of inhibition, despite MRSA's resistance to ampicillin. This finding is consistent with the explanation that the antibiotics acted synergistically when used simultaneously (56). It is possible that the interaction between the antibiotics weakened the resistance of MRSA to the beta-lactam antibiotic class. Further research might determine that the antibiotic produced by *S. violaceorectus* displays superior synergistic drug interactions with other antibiotics.

The mechanism of action of the antibiotic produced by *S. violaceorectus* might have also negatively affected one or more mechanisms of resistance in MRSA. This would explain the increase in susceptibility of MRSA to ampicillin. For example, membrane permeability could have increased, drug efflux could have decreased, or bacterial enzymes could have been inhibited. Therefore, the antibiotic should be of interest to pharmaceutical companies because of its potential to be used in medical

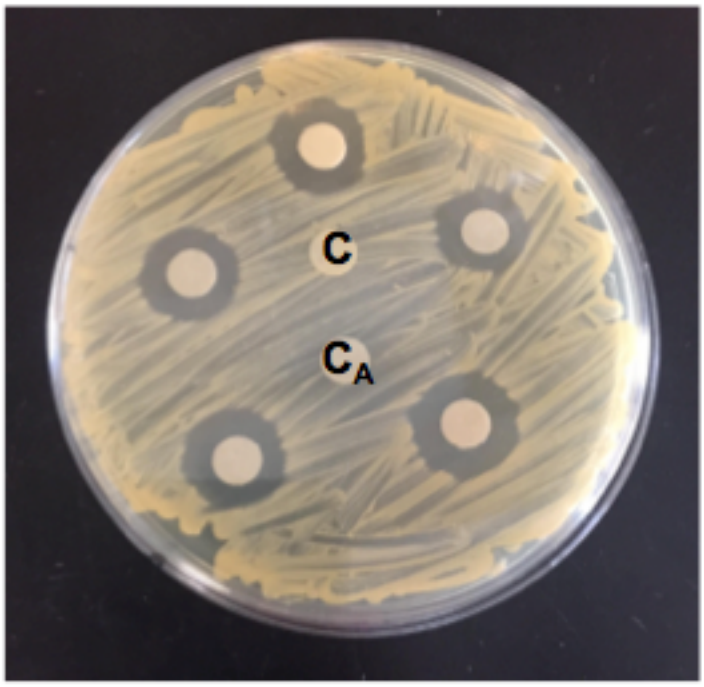
practice as a treatment for infected patients. New discoveries such as those from this study appear promising in the current antibiotic resistance crisis.

Figure 2-3

A.



B.



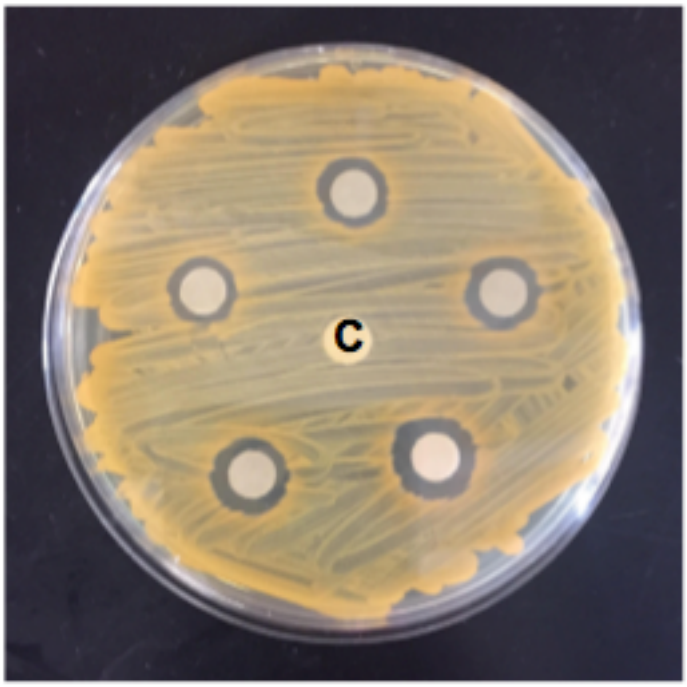
C.

		MRSA Treatment	
		Extract	Extract & Ampicillin
Diameter of Zone of Inhibition (mm)	Disc 1	12.6	14.1
	Disc 2	12.5	13.8
	Disc 3	11.8	15.1
	Disc 4	13.3	14.5
	Disc 5	10.5	14.0
	Disc 6	12.9	12.7
	Disc 7	13.7	13.9
	Disc 8	11.0	14.0
	Disc 9	13.0	12.2
	Disc 10	12.2	13.1
	Disc 11	11.1	14.1
	Disc 12	12.0	15.2
	Disc 13	11.8	13.8
	Disc 14	12.4	14.1
	Disc 15	12.1	14.0
	Average	**12.19**	**13.91**

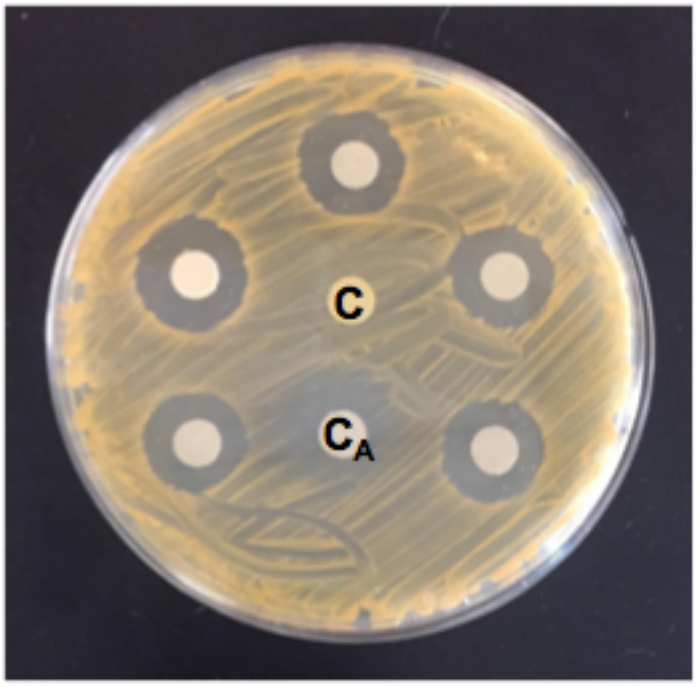
Figure 2-3. Combination antibiotic therapy of antibiotics on MRSA. Disc assays compared the difference in inhibitory effect of the antibiotic produced by *S. violaceorectus* when used alone and in combination with ampicillin on MRSA. **A.** Representative disc assays to test for the ability of the antibiotic produced by *S. violaceorectus* to inhibit the growth of MRSA. The symbol "**C**" represents the methanol control. **B.** Representative disc assays to test for the ability of the combination of ampicillin and the antibiotic produced by *S. violaceorectus* to inhibit the growth of MRSA. The symbol "**C**" represents the methanol control, and the symbol "$\mathbf{C_A}$" represents the ampicillin control. **C.** Measurements of the diameter of the zone of inhibition on 15 disc assays. The antibiotics in combination were more potent against MRSA than the use of only the antibiotic produced by *S. violaceorectus.* This result was significant with a p-value less than 0.00001.

Figure 2-4

A.



B.



C.

		MSSA Treatment	
		Extract	Extract & Ampicillin
Diameter of Zone of Inhibition (mm)	Disc 1	12.7	14.7
	Disc 2	11.2	15.0
	Disc 3	11.2	14.6
	Disc 4	11.5	15.1
	Disc 5	11.4	14.1
	Disc 6	10.8	16.1
	Disc 7	11.0	15.8
	Disc 8	12.3	15.1
	Disc 9	11.1	14.5
	Disc 10	10.2	17.0
	Disc 11	11.8	15.6
	Disc 12	12.4	14.7
	Disc 13	11.1	15.9
	Disc 14	13.8	14.0
	Disc 15	12.5	15.6
	Average	**11.67**	**15.19**

Figure 2-4. Combination antibiotic therapy of antibiotics on MSSA. Disc assays compared the difference in inhibitory effect of the antibiotic produced by *S. violaceorectus* when used alone and in combination with ampicillin on MSSA. **A.** Representative disc assays to test for the ability of the antibiotic produced by *S. violaceorectus* to inhibit the growth of MSSA. The symbol "**C**" represents the methanol control. **B.** Representative disc assays to test for the ability of the combination of ampicillin and the antibiotic produced by *S. violaceorectus* to inhibit the growth of MSSA. The symbol "**C**" represents the methanol control, and the symbol "$\mathbf{C_A}$" represents the ampicillin control. **C.** Measurements of the diameter of the zone of inhibition on 15 disc assays. The antibiotics in combination were more potent against MSSA than the use of only the antibiotic produced by *S. violaceorectus.* This result was significant with a p-value less than 0.00001.

2.4: Limitations

Although the experiments demonstrated that the antibiotic produced by *S. violaceorectus* is more effective against MRSA when combined with ampicillin than when used alone, the structure and mechanism of action of the antibiotic remains unknown. It could be an antibiotic that has been studied before, possibly one that is currently used as a medical treatment. Without knowledge of the molecular nature of the antibiotic, its mechanism of action against MRSA is difficult to determine. This would have narrowed down the possibilities as to the reasons why the synergistic effects with ampicillin were observed. Additionally, the antibiotic produced by *S. violaceorectus* was used only once on any given lawn of MRSA. If there was repeated use, the likelihood of MRSA acquiring resistance to it over time exists and is highly probable. Slight variations in the amount of antibiotic distributed on each disc assay also could have affected the results because the discs were simply dipped into a solution of *S. violaceorectus* extract. To address these issues, further tests need to purify and identify the compound (or compounds) present in *S. violaceorectus* extracts that inhibits the growth of MRSA.

2.5: Future Directions

As the antibiotic resistance crisis continues, it is essential that antibiotic stewardship programs are implemented and new antibiotics are discovered. However, the emergence of novel drugs has declined (61). Many antibiotics are currently in clinical development, whether natural products or chemical derivatives of natural products, but have not been proven to be completely effective against various multidrug-resistant bacterial infections (65). Therefore, the mechanisms of resistance in bacteria must be studied before pharmaceutical companies can demonstrate the efficacy of new drugs. Once understood, existing antibiotics can be chemically modified to help target and combat distinct pathways of resistance (65). For example, species-specific antibiotics can be generated, which would prevent many of the adverse effects of using broad-spectrum antibiotics (66). Additionally, different screening techniques can be employed to search for organisms that produce new antibiotics or possibly new antibiotic classes. Technological advances can explore microbial diversity or utilize hypersensitive approaches to detect antimicrobial agents (65). Novel computational methods to screen for antibiotic synergism, such as INDIGO, can also improve the success of combination antibiotic therapy (58). With the discovery of additional synergistic antibiotic combinations, treatment options for patients will expand and the public health hazard of multidrug-resistant bacterial infections will diminish.

Streptomyces are a current focus of study because of their potential to produce antimicrobial substances (50). For example, transposon mutagenesis has been utilized to transport DNA sequences from antibiotic-producing *Streptomyces* into other

Streptomyces species (67). The transposons generally contain genes for antibiotic production or genes that will up-regulate the biosynthesis of antibiotics (67). This process of transposon mutagenesis can be assisted by the complete genome sequencing of different *Streptomyces* species. With genomic data, the metabolic pathways of antibiotic biosynthesis can be constructed and the genes involved in antibiotic production can be identified (68). Additionally, new bacterial growth strategies, such as co-culturing, can induce antibiotic production in *Streptomyces* (69). Bacteria produce antibiotics in response to their environment in order to protect themselves from natural enemies (4). Thus, the environmental stress that results from culturing multiple bacterial species together can enhance the production of various compounds. Therefore, the future of antibiotic discovery could begin with understanding antibiotic production in *Streptomyces*.

The antibiotic produced by *S. violaceorectus* has demonstrated its potential to be used as a medical treatment for antibiotic-resistant bacterial infections. However, it must first be purified and identified before any further tests are conducted to help determine whether it is a new antibiotic or one that has already been discovered. If novel, then its molecular structure and mechanism of action against MRSA should be studied to determine its effectiveness against different bacterial pathogens. This would also create opportunities for the identification of the pathways in which the antibiotic produced by *S. violaceorectus* can enhance the activity of other antibiotics through combination antibiotic therapy. For example, it should be studied with the INDIGO computational approach (58). Using chemogenomics data, synergistic antibiotic combinations would be predicted and the efficacy of combination antibiotic therapy with the antibiotic

produced by *S. violaceorectus* can be improved. Afterwards and with successful results, there is a possibility for its implementation in clinical trials.

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